

## **A zebrafish model for *Mycobacterium leprae* granulomatous infection**

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1   **Abstract**

2   Understanding the pathogenesis of leprosy granulomas has been hindered by a  
3   paucity of tractable experimental animal models. *Mycobacterium leprae*, which  
4   causes leprosy, grows optimally at ~30°C, so we sought to model granulomatous  
5   disease in the ectothermic zebrafish. We find noncaseating granulomas develop  
6   rapidly, and eventually eradicate infection. *rag1* mutant zebrafish, which lack  
7   lymphocytes, also form noncaseating granulomas with similar kinetics, but these  
8   control infection more slowly. Our findings establish the zebrafish as a facile,  
9   genetically tractable model for leprosy, and reveal the interplay between innate  
10   and adaptive immune determinants mediating leprosy granuloma formation and  
11   function.

12

13   **40 word Significance Statement**

14   The study of leprosy is hampered by the paucity of animal models. This study  
15   describes the development of the genetically tractable zebrafish to study leprosy  
16   pathogenesis, particularly granuloma formation and function.

17

## Introduction

Few animal models exist for the study of *M. leprae* pathogenesis *in vivo*, largely because the  $\geq 37^{\circ}\text{C}$  core temperature of traditional rodent models prevents *M. leprae* survival [1]. *M. leprae* is propagated for research use in the athymic mouse footpad [1], where they induce granuloma formation but not the neurological disease typical of human leprosy [2]. Armadillos develop neurological disease and form granulomas in response to *M. leprae*; however, they do not breed in captivity and lack most genetic, molecular and immunological tools [3]. Cultured macrophages have been used to model early granuloma formation with *M. leprae*, but the scope of this model remains limited [4]. Overall, the host determinants that mediate granuloma formation in leprosy and their role in pathogenesis are incompletely understood.

The zebrafish has become an effective model for studying *Mycobacterium tuberculosis* granulomas using *M. marinum*, the agent of fish tuberculosis, and a close genetic relative of the *M. tuberculosis* complex [5]. *M. marinum* infection of adult zebrafish results in organized, multicentric granulomas that become necrotic, similar to those of human tuberculosis [6]. Zebrafish are housed at  $\sim 30^{\circ}\text{C}$ , similar to the growth optimum of *M. leprae*; indeed, a more than century-old paper reports experimental *M. leprae* infection of several fish species [7]. Therefore, we explored the zebrafish as a leprosy model, with a focus on granuloma development, fate and function.

## Methods

41 Zebrafish husbandry and experiments were conducted at the University of  
42 Washington in compliance with guidelines from the U.S. National Institutes of  
43 Health and approved by the University of Washington Institutional Animal Care  
44 and Use Committee. Four-month old male zebrafish, either wildtype AB strain, or  
45 sibling *rag1*<sup>t26683/t26683</sup> mutants and *rag1*<sup>+/t26683</sup> heterozygotes, were infected  
46 intraperitoneally (as described in [6]) with  $5 \times 10^7$  *M. leprae* isolated from mouse  
47 footpads; bacteria were tested for viability by radiorespirometry, as described [1].  
48 *rag1*<sup>t26683/t26683</sup> and *rag1*<sup>+/t26683</sup> were identified among offspring from a *rag1*<sup>+/t26683</sup>  
49 incross by genotyping using high-resolution melt analysis of amplicons generated  
50 with primers GCGCTATGAGATCTGGAGGA and  
51 TGCAGTGCATCCAGAGTAGG, or GCGCTATGAGATCTGGAGGA and  
52 CAGAGTAGGCTGGGTTTCCA, on a CFX Connect Thermocycler (BioRad).  
53 Animals were observed twice daily and killed by tricaine overdose for each  
54 experimental time point, or in the survival experiment, if they appeared moribund.  
55 To measure bacterial burden, we used histology with Fite staining to detect  
56 bacilli, which is the typical method for diagnosis of human leprosy [8, 9]. Sections  
57 were prepared for histology as described [6]. Briefly, serial sagittal sections were  
58 made from formalin-fixed animals and stained by hematoxylin and eosin to  
59 visualize host cells, and using Fite, a modified acid-fast stain to visualize *M.*  
60 *leprae* which are acid fast bacilli (AFB). Sections were examined using bright  
61 field microscopy and images were collected with a digital photo camera (model  
62 DKC-5000; Sony, Tokyo, Japan) and produced using Metamorph software  
63 (Molecular Devices Corporation, Sunnyvale, CA). Three fish per group per time

point were examined. As a surrogate for bacterial burden per fish, Tissue Studio 4.0 (Definiens) was used to identify the AFB-positive regions in a single sagittal section, and measure their cumulative area. Animals were considered to have cleared infection if no AFB were detected in the entire sagittal section. Serial sagittal sections (3-4 per animal) were examined to confirm there were no significant differences between the sections and that the sections were representative (figure S1A). Statistical analyses were performed using Prism (version 5.0a, GraphPad).

## Results

$5 \times 10^7$  *M. leprae* were injected into zebrafish, similar to the number of bacteria used to inoculate mouse footpads [1]. Within seven days post infection (dpi) with *M. leprae*, zebrafish had formed organized granulomas throughout the body involving the pancreas, liver, intestine, mesentery, blood vessels, gonad and adipose tissue (figure 1A). The granulomas were comprised centrally of macrophages that had undergone epithelioid transformation (characterized by a high cytoplasm to nucleus ratio), with scattered lymphocytes (characterized by a high nucleus to cytoplasm ratio) aggregating at the periphery (figure 1A). Thus, even from this early stage, they resembled the organized granulomas of human leprosy (figure 1B). Fite staining revealed that similar-sized granulomas within the same fish contained varying numbers of bacteria, possibly reflecting ongoing bacterial killing (figure 1C and D).

We sought to determine the role of adaptive immunity in the control of leprosy. For tuberculosis, the critical role of adaptive immunity in the control of infection is highlighted by the role of human immunodeficiency virus (HIV) infection in increasing susceptibility to TB [10]. *rag1* mutant mice lacking mature T and B cells are hypersusceptible to *M. tuberculosis* [5]. Likewise, SCID mice, also lacking mature T and B cells, have increased *M. leprae* burdens in their footpads, which decreases upon administration of T cells to the animals [11]. However, the role of adaptive immunity in the control of human leprosy is unclear. On the one hand, lymphocytes are present in the well-organized granulomas of paucibacillary leprosy, similar to the case with human TB granulomas, and an effective cellular response is associated with paucibacillary leprosy [5, 8]. On the other hand, the evidence that HIV infection exacerbates leprosy in humans is scant, with only isolated reports of increased tendency for multibacillary disease, reactions, and relapse [12].

We previously showed that *rag1* mutant zebrafish are more susceptible to *M. marinum*, recapitulating the findings of *rag1* mutant mice infected with *M. tuberculosis* [5, 6]. Therefore, we asked if *rag1* mutant zebrafish were also more susceptible to *M. leprae*. We compared them to their heterozygous siblings, which are as resistant as wildtype fish to *M. marinum* [6]. By ~60 dpi, the infected mutants had become runted with frayed fins (figure 2A) and began to die soon after (figure 2B). Decreased survival was statistically significant in the infected *rag1* mutants but not the other groups (figure 2B), and all dying animals manifested similar signs of disease before death (runt, frayed fins,

hemorrhaging, and swimming near the tank bottom). Only 3 of 12 infected mutants survived, and these survivors appeared healthy, suggesting some mutants were able to clear infection.

Simultaneously, in a separate small cohort (three *rag1* heterozygote and three mutant animals per time point), we performed tissue histology to assess granuloma morphology and bacterial burdens. *rag1* mutants formed organized epithelioid granulomas by seven days that were similar to wildtype except that, as expected, they lacked lymphocytes (figures 2C). Analysis of Fite-stained histology sections suggested that both heterozygotes and mutants cleared infection over time. At 112 dpi and 168 dpi, two of three *rag1* heterozygotes contained no bacilli, while one of three *rag1* mutants contained no bacilli at those time points (figure 2D; figure S1A.)

In the remaining animals, we assessed bacterial burdens at various time points by quantifying FITE-positive bacteria in multiple sections in each animal (figure S1A). We found that, in the remaining animals, mutant bacterial burdens were greater than heterozygotes at 28 days and then declined (figure 2D). Together, these findings suggest that while adaptive immunity is important in controlling *M. leprae*, it can be controlled by innate immunity alone. Whether these differences reflect differences in bacterial replication, bacterial killing or both, awaits the development of direct assays for bacterial replication *in vivo*.

A curious feature of *M. leprae* granulomas is that they seldom become necrotic, even when laden with organisms [8]; this is in sharp contrast to human tuberculous granulomas [5]. In the zebrafish too, we found that even

multibacillary lesions where individual macrophages were packed with bacteria seldom became necrotic (figure S1B). Necrosis was observed in only 2.9% of heterozygote granulomas (1 of 34 granulomas in 12 animals) (figure S1C). Similarly, only a minority of the *rag1* mutant granulomas became necrotic – 14%, or 7 of 50 granulomas in 12 animals; this difference was not statistically significant.

Finally, human leprosy granulomas are frequently associated with damage to peripheral nerves. We were unable to assess nerve damage in this study, as even an experienced neuropathologist was unable to identify the nerves in these small animals. In a companion study using zebrafish larvae, which are transparent, we have been able to show the association between early macrophage aggregates and nerve injury [13].

## Discussion

This pilot study suggests the promise of the adult zebrafish as a model for studying *M. leprae* granuloma formation and function, and the immune pathways that determine host susceptibility to leprosy. Morphologically, most granulomas resemble those of paucibacillary (or tuberculoid) human leprosy, and like their human counterparts, they are effective in controlling infection [14]. Indeed, the vast majority of humans appear to clear *M. leprae* infection [14], and most zebrafish do as well. As with humans, our data suggest that the ability of zebrafish to clear *M. leprae* infection differs among individuals. This likely reflects varied immune responses in the zebrafish, which, like humans, are outbred (in



contrast, mice are inbred). Dr. Richard Truman at the National Hansen's Disease Programs has found a similarly high degree of fish-to-fish variability when he used *M. leprae* to infect medaka, another outbred fish species (personal communication).

Another intriguing feature of human leprosy is the rarity of granuloma necrosis [8], and this too is preserved in the zebrafish. This could be because *M. leprae* has lost determinants present in *M. marinum* and *M. tuberculosis* that promote granuloma macrophage necrosis.

Finally, our work reveals the complexity of the interplay between innate and adaptive immunity in the control of leprosy. In separate work, we have developed the larval zebrafish as a leprosy model, and we find that macrophages can aggregate into granulomas and control *M. leprae* to a substantial extent in the sole context of innate immunity [13]. Our findings here with the *rag1* mutant reinforce the idea that bona fide epithelioid granulomas form without adaptive immunity [5], yet the full microbicidal capacity of the granuloma macrophages requires stimulation by adaptive immunity. Indeed, we find that lymphocytes begin to arrive in the granuloma by seven days after infection, and bacterial burdens diverge between *rag1* heterozygotes and mutants by 28 days (figure 2D). Thereafter, bacterial burdens drop even in the *rag1* mutant fish, suggesting that innate immune factors can gradually control infection (figure 2D). The finding that mutants slowly reduce bacterial burdens, and occasionally even clear infection, suggests that innate immunity alone may be sufficient to control this slowly growing pathogen. The decreased survival of *rag1* mutants in the face of

178 this delayed control may reflect the adverse consequences of chronic infection,  
179 or be due to cytokine dysregulation in the absence of adaptive immunity. In any  
180 case, our zebrafish findings may reflect the lack of an obvious link between  
181 exacerbation of leprosy and HIV co-infection [12]. Moreover, given that innate  
182 immunity has a role in clearing infection, the development in humans of  
183 multibacillary rather than paucibacillary leprosy may well reflect innate immune  
184 deficiencies, some of which are beginning to be identified [8, 15]. It is our hope  
185 that these can be broadly identified and studied in the zebrafish, using the  
186 publicly available libraries of zebrafish mutants have been generated by chemical  
187 mutagenesis and CRISPR technologies.

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## **Figure Legends**

**Figure 1.** Adult zebrafish are susceptible to *M. leprae* infection. Panel A shows a hematoxylin and eosin (H&E) section of a granuloma in the peritoneal cavity of a wildtype adult zebrafish, 7 days post infection (dpi) with  $5 \times 10^7$  Thai53 strain *M. leprae*. Arrowheads indicate lymphocyte nuclei. In panel B, a granuloma from a skin biopsy of human tuberculoid leprosy patient; Archives of Lauro de Souza Lima Institute. In panel C, a serial section of the granuloma in A, stained for acid-fast bacilli (AFB) to detect *M. leprae*; many bacteria are present (arrows). In panel D, an AFB-stained granuloma section from the peritoneal cavity of a similarly infected fish at 7 dpi; few bacteria are present. Arrows indicate bacilli. 10µm bars.

**Figure 2.** Adaptive immunity contributes to control of *M. leprae* infection. In panel A, representative images of sibling uninfected and infected *rag1* mutant animals ~100 days after infection; the *M. leprae*-infected animal is smaller than the uninfected animal. Arrows indicate an intact fin in the uninfected animal and a frayed fin in the infected animal. In panel B, Kaplan-Meier survival curve of sibling *rag1* heterozygote and mutant zebrafish, infected or not with *M. leprae* as in figure 1A. Number of animals: 61 uninfected heterozygotes, 20 infected heterozygotes, 57 uninfected mutants, 41 infected mutants. In panel C, an H&E-

stained section through a *rag1* mutant zebrafish granuloma, infected as in figure 1A; 10µm bar. In panel D, quantification of bacterial burden per fish in *rag1* heterozygotes and mutants; \*p=0.03, other comparisons not significant; student's T test comparing heterozygotes to mutants at each time point.

**Supplemental Figure 1.** Detailed analysis of *M. leprae* granulomas. In panel A, multiple AFB-stained sections from infected fish at 112 dpi were scored for number of infected granulomas; n, number of sections scored. In panel B, an AFB-stained section of a non-necrotizing granuloma in a *rag1* heterozygote zebrafish with heavily infected macrophages (arrows). In panel C, AFB and H&E sections of a necrotic granuloma observed in *M. leprae*-infected *rag1* heterozygote fish. 10µm bars.

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